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REMARKS

Claims 245 and 275 have been amended to more distinctly claim that which Applicants regard as their invention. As will be discussed below, amended claims 245 and 275 are supported by the specification.

The First Rejection Under 35 U.S.C. 112, First Paragraph

Claims 245-279 stand rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention, for the same reasons of record set forth in the Official action mailed 02/17/99. It is specifically asserted that Applicant's response provides only an assertion that an ordinarily skilled artisan, armed with the specification, could practice the invention without undue experimentation. This assertion, without any supporting evidence, fails to overcome the prima facie case of lack of enablement. The Examiner in the Office Action dated February 17, 1999 has asserted that

> Claims 245-279 re drawn to a "multimeric complex composition" having "monomeric unit(s)" attached via "polymeric interactions." The language "multimeric complex composition" bound via "polymeric interactions" reads on associations of any polymer, ie. Any chemical compound or mixture of compounds combined and consisting of essentially repeating structural units, and therefore reads on nylon or any other non-biological polymeric composition as well as duplex DNA, RNA, etc. The scope of the genus sought for such constructs is not enabled in view of the lack of specificity of guidance in the specification as filed. The specification fails to provide guidance for the breadth claimed since the claims nebulously claim "multimeric compositions." The specification teaches only prophetically nucleic acid constructs having multimeric units bound to binding matrices for cell interaction. The

specification only exemplifies application of constructs for expression of antisense to HIV in which the only "multimeric units" are nucleotides forming duplex DNA "complexes".

Applicants respectfully traverse the rejection. First, in Applicants view, it would not require undue experimentation for the ordinary skilled artisan to practice the invention. A sufficiently detailed description is provided in the specification for obtaining the multimeric complexes of the present invention on pages 69-80. Working examples are provided in Examples 16-18. These include the preparation of a multimeric antibody, preparation of multimeric insulin by hybridization and by hybridization to discrete sequences. These are illustrated in Figures 21, 22 and 23. Applicants attach hereto a decision tree provided with "Training Materials for Examining Patent Applications with Respect to 35 U.S.C. Section 112, First Paragraph-Enablement Chemical/Biotechnical Applications". Two questions are posed in the decision tree. The first is "Does the specification teach how to make and use at least one embodiment encompassed by the claims as a whole without undue experimentation?" Clearly, the specification has taught one of ordinary skill in the art how to make and use more than one embodiment. The second question is "Are the enabled embodiments representative of the full scope of the claims?" Again the answer is yes. The methods described for obtaining the disclosed multimeric complexes could be applied to obtaining any of the multimeric complexes encompassed by the pending claims. Therefore, the scope of the composition claims, 245-266 and 275-279 is appropriate.

It has also been asserted that Applicant does not further address the enablement of the claimed constructs applied to whole organisms as broadly claimed. In the Examiner's view, barriers to successful delivery of antisense to

the organism are: (1) penetration of the plasma membrane of the target cells to reach the target site in the cytoplasm or nucleus (2) withstanding enzymatic degradation and (3) the ability to find and bind the target site and simultaneously avoiding non-specific binding. The Office Action cites passages from Branch and Flanagan as evidence of skepticism of those of skill in the art.

In response, Applicants note that Branch and Flanagan were actually published *after* the priority date of the above-referenced application. The MPEP in Section 2164.05(a) states that "the state of the art existing at the filing date of the application is used to determine whether a particular disclosure is enabling as of the filing date." This section further states "In general, the examiner should not use post-filing date references to demonstrate that the patent is no-enabling." Applicants nevertheless assert that there are a number of publications available as of the priority date of the above-referenced application as well as publications published after the priority date of the above-referenced application which express a more optimistic attitude regarding the suitability of antisense to become useful in therapeutic application. One example of such a publication is Crooke, 1994, Antisense Research and Development 4:145-6, attached hereto as Exhibit 2. Another example is Liu et al, 1997, J. Virol. 71:4079-4085, attached hereto as Exhibit 3 which discloses Tat-activated expression of chloramphenicol acetyltransferase was shown to be specifically inhibited in cells expressing Tat and transactivation response region antisense sequences.

It is also Applicants' position that *in vivo* data is not necessary. As noted in the MPEP Section 2107.03, III, "Office personnel should be careful not to find evidence unpersuasive simply because no animal model for the human disease condition had been established prior to the filing of the application". However,

Applicants do note that clinical trials were being conducted by the assignee of the instant application around its priority date (Exhibit 4). The results have been favorable and a number of public announcements have been made concerning the ongoing clinical trials and results.

Applicants would also like to respond to other points raised in the Office Action. First, as conceded in the Office Action, Applicants have demonstrated the penetration of cells by the antisense compounds, notably antisense inhibition of HIV in infected U937 cell culture. Therefore, the question of penetration of the plasma membrane of target cells should not be an issue.

Second, Applicants note that specificity to any degree and certainly 100% specificity is not required of any drug under the patent laws and is evaluated on a case-by-case basis by the Food and Drug Administration. For example, penicillin is known to be far from specific to a certain target protein of harmful bacteria. However, this does not diminish the importance of penicillin as a useful drug.

The Second Rejection under 35 U.S.C. §112, First Paragraph

Claims 245-266 stand rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. In the Office Action dated February 19, 2001, the Examiner stated:

> Claims 245-266 are drawn to a "multimeric complex composition" having "monomeric unit(s)" attached via "polymeric interactions."
>
> The claims broadly encompass "constructs" for producing a "product" and it is not clear what is embraced by the claims. The

> claims read on vectors, genomes, cell processes like translation, transcription, etc....Claims 245-266 read on any polymer composition.
> The instant specification describes prophetically a number of potential modified nucleic acid constructs for expression of an entity in a cell. The supporting figures provide limited additional disclosure of relevant identifying structural characteristics because they primarily correspond to expression vector based constructs which are only one facet of the invention in light of the nebulous scope claimed.
> Clearly the specification only considers vector-like constructs for delivery and expression of nucleic acids. Specifically, for claims 245-266, the only "multimeric compositions" exemplified are to those for antisense inhibition of HIV.

Applicants respectfully traverse the rejection. The Final Written Description Guidelines state in Paragraph II.A.3.a.

> Possession may be shown in many ways. For example, possession may be shown, inter alia, by describing an actual reduction to practice of the claimed invention. Possession may also be shown by a clear depiction of the invention in detailed drawings or in structural chemical formulas which permit a person skilled in the art to clearly recognize that applicant had possession of the claimed invention. An adequate written description of the invention may be shown by any description of sufficient, relevant, identifying characteristics so long as a person skilled in the art would recognize that the inventor had possession of the claimed invention. . .
> An applicant may show possession of an invention by disclosure of drawings[39] or structural chemical formulas[40] that are sufficiently detailed to show that applicant was in possession of the claimed invention as a whole. The description need only describe in detail that which is new or not conventional.[41] This is equally true whether the claimed invention is directed to a product or a process.
> An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics[42] which provide evidence that applicant was in possession of the claimed invention,[43] i.e., complete or partial structure, other physical and/or chemical properties, functional

characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.[44] What is conventional or well known to one of ordinary skill in the art need not be disclosed in detail.[45] If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met.[46]

The Written Description Guidelines further state in paragraph II.A.3.a.(2)

(2) For each claim drawn to a genus:
The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice ....., reduction to drawings ....., or by disclosure of relevant, identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus (citation omitted).
A ``representative number of species'' means that the species which are adequately described are representative of the entire genus. Thus, when there is substantial variation within the genus, one must describe a sufficient variety of species to reflect the variation within the genus. On the other hand, there may be situations where one species adequately supports a genus (citation omitted). What constitutes a ``representative number'' is an inverse function of the skill and knowledge in the art.
Satisfactory disclosure of a ``representative number'' depends on whether one of skill in the art would recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed. For inventions in an unpredictable art, adequate written description of a genus which embraces widely variant species cannot be achieved by disclosing only one species within the genus (citation omitted).

> Description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces (citation omitted). If a representative number of adequately described species are not disclosed for a genus, the claim to that genus must be rejected as lacking adequate written description under 35 U.S.C. 112, para. 1.

Applicants assert that an adequate description has been provided. A detailed description of the multimeric complexes and compositions of the present invention. Additionally, the term "monomeric units" and "polymeric interactions" are also clearly defined. In particular, as noted above, A sufficiently detailed description is provided in the specification for obtaining the multimeric complexes of the present invention on pages 69-80. Working examples are provided in Examples 16-18. These include the preparation of a multimeric antibody, preparation of multimeric insulin by hybridization and by hybridization to discrete sequences. These are illustrated in Figures 21, 22 and 23.

The disclosures in the specification clearly conform to the Written Description guidelines. A depiction of the invention has certainly been provided in Figures 21, 22 and 23. Applicants note that three cases are cited in footnote 39 pertaining to the use of drawings pertaining to the adequacy of the Written Description Requirement. Specifically, footnote 39 states

> See, e.g., *Vas-Cath*, 935 F.2d at 1565, 19 USPQ2d at 1118 (``drawings alone may provide a `written description' of an invention as required by Sec. 112''); *In re Wolfensperger*, 302 F.2d 950, 133 USPQ 537 (CCPA 1962) (the drawings of applicant's specification provided sufficient written descriptive support for the claim limitation at issue); *Autogiro Co. of America v. United States*, 384 F.2d 391, 398, 155 USPQ 697, 703 (Ct. Cl. 1967) (``In

> those instances where a visual representation can flesh out words,drawings may be used in the same manner and with the same limitations as the specification.").

Sufficient identifying characteristics of the constructs, compositions and kits of the present invention is provided as noted above in the specification. Additionally, a sufficient number of species have been disclosed. Finally, Applicants note that actual reduction to practice is not required to satisfy the Written Description Requirement. Footnote 36 of Written Description Guidelines state

> ....."The word 'invention' must refer to a concept that is complete, rather than merely one that is 'substantially complete.' It is true that reduction to practice ordinarily provides the best evidence that an invention is complete. But just because reduction to practice is sufficient evidence of completion, it does not follow that proof of reduction to practice is necessary in very case. Indeed, both the facts of the *Telephone Cases* and the facts of this case demonstrate that one can prove that an invention is complete and ready for patenting before it has actually been reduced to practice.

Therefore, the claimed invention is adequately described.

In view of the above arguments, Applicants assert that the rejection has been overcome. Applicants therefore request that the rejection under 35 U.S.C. 112, first paragraph (written description) be withdrawn.

The Rejections Under 35 U.S.C. 102(e)

Claims 245-279 stand rejected under 35 U.S.C. §102(e) as being anticipated by Curiel et al. ("Curiel"), for the same reasons of record set forth in the Official action mailed 02/17/99. It is asserted that Applicant does not address how the teaching of Curiel does not read on the instant claims as broadly drawn to multimeric complexes.

Claims 245-266 and 275 stand rejected under 35 U.S. C. §102(e) as being anticipated by Edwards et al. ("Edwards"), for the same reasons of record set forth in the Official action mailed 02/17/99. It is asserted that Applicant does not address how the teaching of Edwards does not read on the instant claims as broadly drawn to multimeric complexes.

Claims 245-279 have been rejected under 35 U.S.C. §102(e) as being anticipated by Paul ("Paul"), for the same reasons of record set forth in the Official action mailed 02/17/99. It is asserted that Applicant does not explain how the elements taught by Paul are not material to the instant invention as broadly claimed.

Applicants respectfully traverse each of the rejections. Invalidity for anticipation requires that all of the elements and limitations of the claim are found within a single prior art reference. There must be no difference between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of the invention. *Scripps Clinic & Research Foundation v. Genentech Inc. 927 F.2d 1565, 18 USPQ2d 1001, 18 USPQ2d 1896 (Fed. Cir. 1991).* Applicants will address each of the rejections below.

Curiel

The multimeric complex compositions of the present invention can be distinguished from the conjugate of Curiel. No multimeric complex is disclosed in Curiel Specifically, the conjugate of Curiel comprises a virus bound via an antibody to a substance such as a polycation, which binds to a nucleic acid. In contrast to the multimeric complex of the present invention, the antibody and virus of Curiel are in monomeric form (see Figure 1 of Curiel-attached). The lysines in the polycation are bound to one another via peptide bonds (covalent).

However, as described in Example 17 of the instant application, in the embodiment using antibodies, the antibodies are in multimeric form. A multimerization procedure is shown in Figure 21 of the instant specification. The antibodies are attached to each other via noncovalent interactions. Clearly, the complexes of the present invention can be distinguished from the complexes disclosed by Curiel Therefore, the compositions of the present invention and methods of using said compositions can be distinguished from the conjugates and complexes of Curiel

Edwards

The multimeric complex compositions of the present invention can be distinguished from the system disclosed by Edwards. The assay system for detecting the binding of small molecules to DNA test sequences comprised: (a) the DNA binding small molecule which may be in homopolymeric or heteropolymeric form which competes with a DNA binding protein for binding to (b) an oligodeoxynucleotide that contains the recognition sequence for the DNA-binding protein flanked on either or both sides by a variable test site. As described in column 8, lines 20-42 of Edwards, the monomeric units in the homopolymers and heteropolymers are coupled together via covalent bonds. In contrast, amended claims 245 and 275 in the instant application recite that monomeric units are attached to each other through noncovalent polymeric interactions, specifically through polymeric interactions of the polymers of the monomeric units.

Additionally, Applicants note that it is disclosed in Edwards that the small molecule may be functionally bound to the DNA molecule, specifically by the noncovalent association of a protein or small molecule to the DNA molecule.

There is no disclosure in Edwards regarding a polymer attached to such a small molecule. Actually, the premise of Edwards is that the small itself should bind to a specific DNA sequence. In contrast, in the composition of the present invention, it is the polymeric element of the monomeric unit that binds to the polymeric element of the binder.

In view of the amendment of claims 245 and 275 and the above arguments, Applicants assert that the compositions of the present invention can be distinguished from the assay system disclosed by Edwards. Therefore, Applicants respectfully request that the rejection of the claims over Edwards be withdrawn.

Paul

The multimeric complex compositions of the present invention can be distinguished from the vectors disclosed by Paul. No multimeric complex is disclosed in Paul. Specifically, Paul discloses retroviral particles having chimeric targeting proteins capable of binding to specific target sequences, such as receptors on the cell. The chimeric targeting proteins contain a ligand moiety, which is derived from a cytokine, and an uptake moiety derived from a retroviral envelope protein. There does not appear to be any teaching regarding the formation of multimeric complexes of the chimeric targeting proteins. Furthermore, neither component of the chimeric protein taught by Paul appears to be in polymeric form. Furthermore, the receptor disclosed by Paul is not in polymeric form.

In contrast, the multimeric complex compositions of the present invention as in amended claim 245 and 275 recite that the monomeric units comprise a compound and polymer and that the binding matrix comprises a polymer.

Furthermore, in the complexes of the present invention, the monomeric unit binds to another monomeric unit through noncovalent polymeric interaction and the monomeric unit attaches to the binder through noncovalent polymeric interactions between the polymeric component of the monomeric unit and polymer of the binding matrix. Paul discloses no such interactions.

In view of the amendments to claims 245 and 275, and the above arguments, Applicants assert that the compositions of the present invention can be distinguished from the assay system disclosed by Paul. Therefore, Applicants respectfully request that the rejection of the claims over Paul be withdrawn.

* * * * * * *

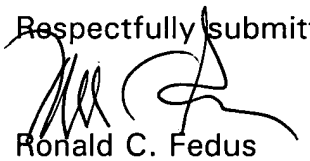
SUMMARY AND CONCLUSIONS

Claims 245-279 are presented for further examination. Claims 245 and 275 have been amended. No claims have been added by this paper.

No claim fee or other fees are believed due in connection with this response. In the event that any fee is due for this paper or any paper being filed in connection with the accompanying Petition, however, The Patent and Trademark Office is authorized to charge any such fee or fees to Deposit Account No. 05-1135, or to credit any overpayment thereto.

If a telephone conversation would further the prosecution of the present application, Applicants' undersigned attorney requests that he be contacted at the number provided below.

Respectfully submitted,



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MARKED UP VERSION OF THE CLAIMS

245. (Amended) A multimeric complex composition comprising more than one monomeric unit, said monomeric unit comprising a compound and polymer and wherein said monomeric units are attached

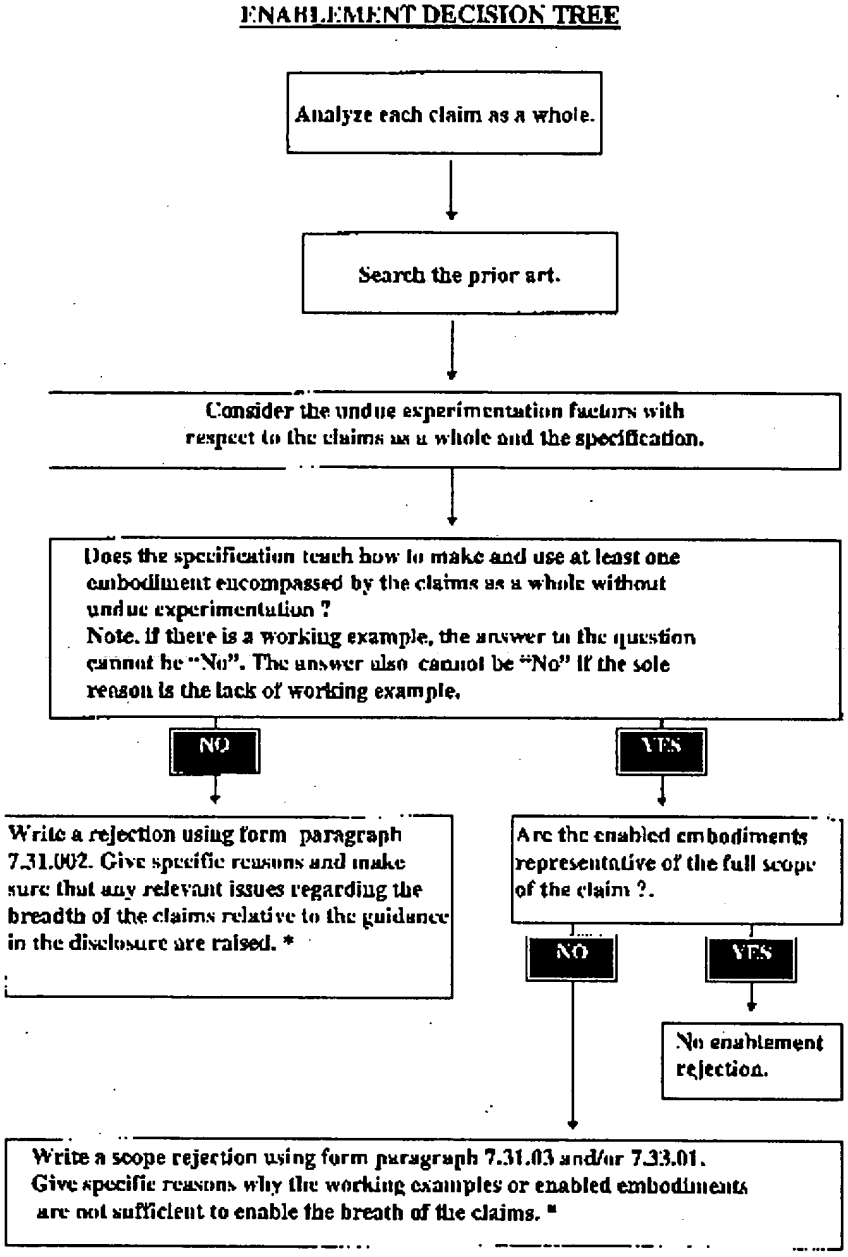
(a) to each other through [noncovalent] polymeric interactions of the polymers of the monomeric units, or

(b) to a binding matrix comprising a polymer through noncovalent polymeric interactions between said polymer of said monomeric unit and said polymer of said binding matrix, or

(c) both (a) and (b).

275. (Amended) A multimeric composition comprising more than one component attached noncovalently to a charged polymer, wherein said charged polymer is selected from a polycationic polymer, a polyionic polymer, a polynucleotide, a modified polynucleotide and a polynucleotide analog, or a combination of the foregoing.

* * * * * * *



Examp * If specific technical reasons cannot be given or properly supported with sufficient evidence, then the answer to the previous question should have been "yes".

Example A: Hybridization Probes I

Specification: The specification discloses that bacteria A is known to cause a specific disease and, therefore, detection of bacteria A in a sample is desirable. The specification even discloses that methods are known which detect bacteria A in a sample via culturing techniques. According to the specification, such detection methods are difficult to perform and therefore detection methods using nucleic acid probes are preferred.

The specification discloses that one object of the invention is to provide nucleic acids complementary to unique nucleic acid sequences within the RNA or DNA of bacteria A and which can be used to detect bacteria A. Another object of the invention is to provide a method of detecting bacteria A in a sample by contacting the sample with a probe which preferentially hybridizes to RNA or DNA of

ANTISENSE RESEARCH AND DEVELOPMENT 4:145–146 (1994)
Mary Ann Liebert, Inc., Publishers

Editorial

Progress in Evaluation of the Potential of Antisense Technology

THE CENTRAL QUESTION ABOUT ANTISENSE TECHNOLOGY has never been whether the concept is exciting, but rather whether it would work. This question has been asked in many vernaculars depending on the background of the person asking the question, but it reduces to the issue of whether oligonucleotides will have satisfactory drug properties. Will they have appropriate pharmacologic, toxicologic, and pharmacokinetic properties to realize the potential of the antisense mechanism, and is there sufficient scope for medicinal chemistry to generate new analogs with improved properties? Although definitive answers to questions about the value of drugs of any sort must await broad clinical use after marketing, the answers we have today persuasively argue that the technology should be vigorously pursued and rigorously evaluated and may work as we hope. Virtually all the data that support this view derive from studies on phosphorothioates, but exciting new chemical classes are being tested and a number are in animal studies today.

Recently, we reported on the results of Phase I/II studies of intravitreally administered ISIS 2922 in AIDS patients with refractory CMV retinitis (Crooke, 1994; Palestine *et al.*, 1994). This study demonstrated that ISIS 2922 produced a dose-dependent inhibition of progression of CMV retinitis in patients who had failed all other CMV therapy, had median CD4 counts of 4, and had a median duration of CMV retinitis of 11 months. This study also demonstrated that the drug could be given once every other week with maintenance of prolonged remissions. The main drug-related adverse event was exacerbation of ocular inflammation. Armed with this information, we are initiating definitive clinical trials for this drug. This study is exciting because it is the first study to demonstrate clinical activity of an antisense oligonucleotide and because the drug resulted in rapid, meaningful responses in desperately ill patients with virtually no other recourse. Of course, we have not yet defined the *value* of ISIS 2922 in this disease. That awaits the completion of more definitive trials. Nor does this study guarantee that ISIS 2922 will be approved as therapy for this disease. Nor can be we prove that the principal mechanism of action of ISIS 2922 in this study is antisense. Further, the study was not designed to show that antisense oligonucleotides are active when administered systemically to humans. Nevertheless, taken in the context of all other available data, these results are very encouraging.

We have also recently reported data suggesting that ISIS 2105 when injected intradermally has apparent activity in both primary and surgical adjuvant therapy of genital warts. Again, we must do much more work before we know whether ISIS 2105 is indeed active or valuable in this disease, but we are encouraged by these data as well and are initiating a multiple dose surgical adjuvant Phase II trial to confirm the activity of the drug and determine whether it has sufficient value to be commercialized.

We have reported definitive pharmacokinetic studies on ISIS 2105 in rats after intravenous and intradermal doses (Cossum *et al.*, 1993, 1994). These studies clearly demonstrate excellent bioavailability, peripheral tissue distribution, and clearance that support once a day or every other day dosing. Similar results have been reported by the group at Dupont Merck (Sands *et al.*, 1994), and they have shown autoradiographic results showing drug inside cells in the liver and kidney. We and our colleagues at Ciba-Geigy have similar autoradiographic data, not yet published. Furthermore, we will shortly report definitive pharmacokinetic studies after intradermal dosing in man confirming that man and rats (as well as monkeys, mice, and rabbits) handle phosphorothioates similarly (Crooke *et al.*, 1994). These data are extremely important as they demonstrate attractive parenteral pharmacokinetic properties for phosphorothioate antisense drugs and show that for this class of drugs *in vitro* cell uptake studies do not predict *in vivo* behavior. This last point is not surprising as there is no class of drugs of which I am aware whose pharmacokinetic properties can be simply extrapolated from *in vitro* studies.

In a wide range of studies performed in our laboratories, Hybridon's and others, we have also defined the toxic liabilities of phosphorothioates. We believe the dose limiting toxicities will likely be related to effects on clotting, complement activation, or possibly cytokine release, and that the therapeutic index will be satisfactory. We will also shortly report studies that define the mechanisms underlying these effects.

Perhaps most importantly, however, we and many other laboratories have demonstrated potent systemic effects of phosphorothioates in animals in which all of the data are consistent with an antisense mechanism (Hijiya *et al.*, 1994; Skorski *et al.*, 1994). In our laboratories and those of our collaborators, we have shown potent antisense activities against Ha-RAS, Ki-RAS, PKC-α, RAF kinase, ICAM-1 and other targets. In several cases, we have unequivocally proven mechanism by showing a selective loss of target RNA in various tissues at doses ranging from less than 1 mg/kg to 20 mg/kg daily. Interestingly, because the cells that expressed many of the targets listed above did not take up sufficient oligonucleotide, we had to employ cationic lipid transfection to show *in vitro* activity. *In vivo, no*

specialized delivery system was required. In a particularly important series of studies, Dean *et al.* (1994) have shown potent systemic isotype selective loss of PKC-α RNA induced by a phosphorothioate oligonucleotide and shown 24-hour duration of effects and absence of tachyphylaxis.

In aggregate, all of the data encourage cautious optimism.

So does this mean that the "bullets are really magic"? In my view, this question epitomizes one of the causes of cynicism regarding antisense technology. We are developing a new pharmacological and chemical class of drugs. That we are simultaneously creating a new technology and trying to develop drugs from this technology is entirely appropriate and the only real way to understand the drug properties of these molecules. We hope these drugs will be of unique value. They are, nevertheless, drugs. We expect them to have a variety of effects, but if we can understand these properties in the context of modern pharmacology and antisense drugs continue to perform as well as they have to date, then patients will benefit. With these drugs, as with all other classes of drugs, there will be questions that cannot be answered. However, we have already generated more *direct* proof of mechanism of action in animals than for many more established classes of drugs, and the pharmacokinetic and toxicologic properties appear, at present, to be reasonably attractive.

That there are questions that we cannot answer definitively should not be cause for despair. Do we know the precise mechanisms that explain aspirin uptake into cells? Do we understand, at a biochemical level, how aspirin disproportionates between serum protein binding sites and peripheral tissues? For how many classes of drugs do we have unequivocal direct proof of mechanism of action in animals or man?

I would urge continuing critical evaluation of antisense technology. We need to continue to try to understand these drugs. We expect that we will find limits to their utility. For example, we already know that phosphorothioates do not cross an intact blood brain barrier and are minimally orally bioavailable. We may even unearth effects that negate the potential of this technology entirely. However, the data to date are encouraging, and the technology has successfully overcome a large number of hurdles in a relatively short time.

On the other hand, I would hope for an end to the cynicism about antisense technology. This begins with asking the right questions in the right way. The right questions pertain to factors influencing therapeutic index and the breadth and ease of therapeutic use. The right way to ask the questions is in the context of modern pharmacology and in carefully controlled experiments in which dose response curves for various effects are critically defined.

In short, we must set reasonable expectations for this technology, evaluate its potential reasonably and report our results with integrity. If we do this, we will meet our responsibilities to the technology, to patients in need, and, for those of us in commercial organizations, to investors.

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Stable Human Immunodeficiency Virus Type 1 (HIV-1) Resistance in Transformed CD4$^+$ Monocytic Cells Treated with Multitargeting HIV-1 Antisense Sequences Incorporated into U1 snRNA

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We have approached the development of a human immunodeficiency virus type 1 (HIV-1) therapeutic product by producing immune cells stably resistant to HIV-1. Promonocytic CD4$^+$ cells (U937) were made resistant to HIV-1 by the introduction of a DNA construct (pNDU1A,B,C) that contained three independent antisense sequences directed against two functional regions, transactivation response and *tat/rev*, of the HIV-1 target. Each sequence was incorporated into the transcribed region of a U1 snRNA gene to generate U1/HIV antisense RNA. Stably transfected cells expressed all three U1/HIV antisense transcripts, and these transcripts accumulated in the nucleus. These cells were subjected to two successive challenges with HIV-1 (BAL strain). The surviving cells showed normal growth characteristics and have retained their CD4$^+$ phenotype. In situ hybridization assays showed that essentially all of the surviving cells produced U1/HIV antisense RNA. No detectable p24 antigen was observed, no syncytium formation was observed, and PCR-amplified HIV *gag* sequences were not detected. Rechallenge with HIV-1 (IIIB strain) similarly yielded no infection at a relatively high multiplicity of infection. As a further demonstration that the antisense RNA directed against HIV-1 was functioning in these transfected immune cells, Tat-activated expression of chloramphenicol acetyltransferase was shown to be specifically inhibited in cells expressing Tat and transactivation response region antisense sequences.

The incidence of human immunodeficiency virus (HIV) infection has reached alarmingly high levels worldwide, prompting efforts to develop effective treatments. Much of the development has focused on an antiviral drug-based approach designed for the most part to slow viral growth. We have approached the development of an effective therapeutic product by focusing on rendering the immune cells refractory to HIV type 1 (HIV-1) infection. This will permit reconstitution of a population of immune cells in the presence of HIV-1 and may act to restore immunocompetence. This treatment can be implemented by ex vivo treatment of hematopoietic stem cells to add specific genetic material (4, 13).

As a means of inducing resistance to HIV-1, monocytic cells in culture were transfected with a DNA construct expressing three different sequences, each directed against a critical HIV-1 target sequence. This multitargeting approach was used in order to combat the high rate of variability and mutability of HIV-1 that often results in resistance to therapies against a single virus target (6, 19). The anti-HIV-1 sequences were incorporated into U1 snRNA, a stable and abundant snRNA molecule that functions in RNA processing (3, 23), to generate U1/HIV antisense RNA. U1 snRNA transcripts are synthesized in the nucleus and transported to the cytoplasm, where they undergo modifications; after binding of specific proteins, they are reimported to the nucleus, where they function with other snRNPs in RNA splicing (23). Due to the central role in RNA processing played by U1 snRNA, the U1 promoter is expressed in all cell types. Approximately 10^6 copies of U1 RNA are transcribed from approximately 30 U1 genes per cell. Each promoter thus transcribes an average of approximately 3×10^4 copies per generation (3).

We report here the results obtained by using independent multitargeting U1/HIV antisense constructs to confer stable HIV-1 resistance to CD4$^+$ cells.

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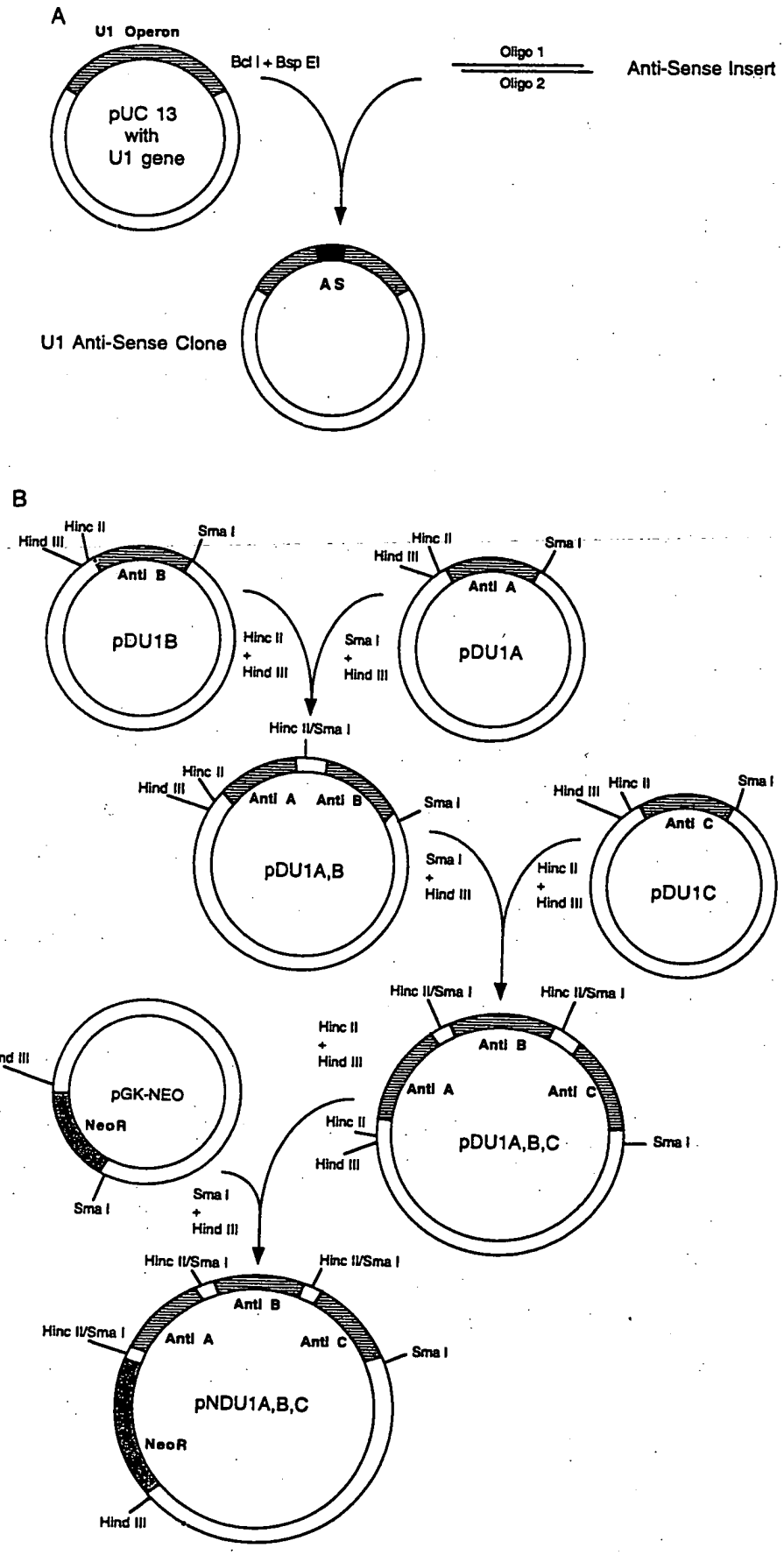
MATERIALS AND METHODS

Construction of plasmids with antisense sequences introduced into the transcript region of the U1 gene. The steps used are presented in Fig. 1. The U1 gene used is derived from pHSD-4 (10). Four pairs of oligonucleotides, designated HVA-1 and HVA-2, HVB-1 and HVB-2, HVC-1 and HVC-2, and HVD-1 and HVD-2, were synthesized. Each pair was hybridized to form double-stranded molecules containing termini compatible with the *BclI* and *Bsp*E1 ends produced from restriction enzyme digestion of the U1 transcript region of the plasmid. Sequence A was taken from the HIV-1 transactivation response region of the HXB2 strain, a T-cell-tropic clone of isolate IIIB (2), sequence B was taken from the tat/rev exon of the LA1 strain, also a T-cell-tropic isolate (5), and sequence C was taken from the tat/rev splice acceptor sequence of the BAL strain, a monocytotropic isolate. Sequence D contains sequences unrelated to either U1 or HIV and serves as a negative control. Sequences of the oligonucleotides are as follows: HVA-1, 5′ GAT CCG GAT TGA GGC TTA AGC AGT GGG TTC CCT AGT TAG CCA GAG AGC TCC CAG GCT CAG ATC TGG TCT AAT 3′; HVA-2, 5′ CCG GAT TAG ACC AGA TCT GAG CCT GGG AGC TCT CTG GCT AAC TAG GGA ACC CAG TGC TTA AGC CTC AAT CCG 3′; HVB-1, GAT CCG GAC CTT GAG GAG GTC TTC GTC GCT GTC TCC GCT TCT TCC TGC CAT AGG AGA GCC TAA GGT 3′; HVB-2, 5′ CCG GAC CTT AGG CTC TCC TAT GGC AGG AAG AAG CGG AGA CAG CGA CGA AGA CCT CCT CAA GGT CCG 3′; HVC-1, 5′ GAT CCG GAT GGG AGG TGG GTC TGA AAC GAT AAT GGT GAG TAT CCC TGC CTA ACT CTA TTC ACT AT 3′; HVC-2, CCG GAT AGT GAA TAG AGT TAG GCA GGG ATA CTC ACC ATT ATC GTT TCA GAC CCA CCT CCC ATC CG 3′; HVD-1, 5′ GAT CAG CAT GCC TGC AGG TCG ACT CTA GAC CCG GGT ACC GAG CTC GCC CTA TAG TGA GTC GTA TTA T 3′; and HVD-2, 5′ CCG GAT AAT ACG ACT CAC TAT AGG GCG AGC TCG GTA CCC GGG TCT AGA GTC GAC CTG CAG GCA TGC T 3′.

BclI and *Bsp*E1 restriction enzyme digestion of pUC13 carrying the U1 operon removes a 41-bp segment (bases 31 through 71) from the transcribed region of


A
U1 Operon
pUC 13
with
U1 gene
Bcl I + Bsp EI
Oligo 1
Oligo 2
Anti-Sense Insert
AS
U1 Anti-Sense Clone
B
Hinc II
Hind III
Sma I
Anti B
pDU1B
Hinc II
+
Hind III
Sma I
+
Hind III
Anti A
pDU1A
Hinc II/Sma I
Anti A
Anti B
pDU1A,B
Anti C
pDU1C
pGK-NEO
NeoR
Anti C
pDU1A,B,C
pNDU1A,B,C

the U1 gene. Each of the hybridized oligomer pairs was inserted into the *BclI*/*Bsp*E1 site created in the U1 transcript region of pHSD-4 to yield plasmids designated pDU1A, pDU1B, pDU1C, and pDU1D. A segment carrying the neomycin resistance gene was derived from pGK-neo (10) and introduced into each plasmid (Fig. 1). The resulting plasmids were designated pNDU1A, pNDU1B, pNDU1C, and pNDU1D. The three U1/HIV antisense cassettes derived from pDU1A, pDU1B, and pDU1C were assembled into a single construct (pNDU1A,B,C) as shown in Fig. 1.

U937 cells carrying U1/HIV antisense constructs. U937 cells (9) were transfected with pNDU1A,B,C by the Lipofectin procedure (Bethesda Research Laboratories [BRL]). The treated cells (5×10^5) were inoculated into T-25 flasks (Corning) containing culture medium (Dulbecco modified Eagle medium; Gibco and BRL) supplemented with 10% heat-inactivated fetal bovine serum (Gibco and BRL) and 600 μg of G418 (Gibco and BRL) per ml. The G418-containing medium was replaced every 3 to 4 days. The resulting population of G418-resistant U937 cells was designated 2.10.16. Selection of a population of cells carrying pNDU1D (designated 2.2.78) was done by using Lipofectin and enrichment for G418 resistance as described above for 2.10.16.

PCR in situ hybridization for HIV-1 DNA sequences. PCR in situ hybridization assays for U1/HIV antisense RNA were performed as described by Nuovo et al. (14, 16). Cells obtained from the late stage of the growth phase were fixed for 15 to 24 h in 10% buffered formalin and then washed twice in diethyl pyrocarbonate-containing water. Approximately 5,000 cells were placed on a silane-coated microscope slide and digested in 2 mg of pepsin per ml at 37°C for 30 min. Assays for HIV-1 DNA were performed by using hot-start PCR (35 cycles) employing the gag primers SK38 and SK39 or SK145 and SK431 (nucleotides 1369 to 1395 and 1507 to 1481). PCR in situ hybridization for the provirus was done with digoxigenin-labeled probes (SK19 and SK102) to assay for viral entry after challenge.

In situ RT-PCR assay for U1/HIV antisense RNA sequence. The procedure described above for in situ PCR detection of DNA was modified for specific detection of U1/HIV antisense RNA sequences. Cells fixed to silane-coated glass slides were protease treated and incubated overnight in RNase-free DNase solution (10 U/section; Boehringer Mannheim) at 37°C to eliminate nonspecific DNA repair and mispriming. This allowed for target-specific direct incorporation of digoxigenin-dUTP (used at a concentration of 10 μM in the reverse transcription [RT]-PCR mix). Primers specific for U1/HIV antisense RNA sequences were used. Assays for U1/HIV-1 RNA sequences used primer HU1, which comprises a sequence from the transcript region of U1 (5′-CCTGGCAGGGG AGATACCATG-3′), as the upstream primer and either HVA-2, HVB-2, or HVC-2 as the downstream primer. For cells transfected with HVA, the HVB-2 and HVC-2 primers served as negative controls; similarly, for HVB transfectants, HVA-2 and HVC-2 served as controls, and for HVC transfectants, HVA-2 and HVB-2 served as negative controls. After protease and DNase digestion, RT and PCR were accomplished by using an rTth EZ kit from Perkin-Elmer as previously reported (15). Pooled normal lymphocytes infected with HIV-1 isolate IIIB for 4 days, and sham-infected cells (kindly provided by Roy Steigbigel, State University of New York at Stony Brook) served as additional controls. The negative and positive controls consistently yielded the expected results. The signal is manifested as a blue precipitate due to the action of the antidigoxigenin-alkaline phosphatase conjugate on the chromogen, nitroblue tetrazolium, and the substrate, β-chloroindolylphosphate. Negative cells stained pink due to the counterstain, nuclear fast red.

Assay for HIV-1 DNA. PCR was performed as described by Schnittman et al. (18), using primer pairs SK38 and SK39 (*gag*). Reaction products were analyzed by electrophoresis in 1.5% agarose in the presence of ethidium bromide. Comparisons were made with amplified DNA from U1.1A, a chronically infected promonocyte cell line which contains two integrated HIV copies per cell (8). U1.1A cells and U937 cells were mixed in various proportions so that in all assays, DNA from 10^5 cells was analyzed.

Transient expression assays for the effect of U1/HIV antisense RNA on Tat-activated expression of CAT. (i) Plasmids. The HIV-1 long terminal repeat-chloramphenicol acetyltransferase (CAT) reporter construct pU3III-CAT was a gift from Craig Rosen (17). The Tat plasmid pCV-1 contains a 1.8-kb fragment of HIV-1 cDNA encompassing the tat gene (1).

(ii) DEAE-dextran transfection and CAT assay. In each transfection, 2.5×10^6 cells per condition were used. The cells were washed with serum-free RPMI 1640 (sfRPMI) and resuspended in 1 ml of sfRPMI containing 400 μg of DEAE-dextran (Sigma) in 50 mM Tris-HCl (pH 7.3) with 2.5 μg of total plasmid per condition. Cells were then incubated at 37°C for 1 h, washed with sfRPMI, resuspended in fresh culture medium, and incubated at 37°C for 40 to 45 h. The cells were harvested, washed, and resuspended in 100 μl of 0.25 M Tris-HCl (pH 7.8), and cell extracts were prepared by three cycles of freezing (dry ice-ethanol) and thawing (37°C water bath). Extracts were centrifuged, and the protein content in the supernatants was estimated by using a micro bicinchoninic acid reagent kit (Pierce, Rockford, Ill.).

(iii) CAT activity assay. CAT activity was determined by incubating 25 μg of cell extracts with [^{14}C]chloramphenicol (57.9 mCi/mmol; NEN) and 2.5 mM acetyl coenzyme A (Pharmacia) at 37°C for 2 h. Incubation was followed by extraction with ethyl acetate and ascending thin-layer chromatography. Chromatograms were autoradiographed, and areas of radioactivity were cut out and counted in scintillation fluid for quantification of CAT expression.

HIV-1 challenge assays. Cells (0.5×10^6 cells) were incubated with the BAL or IIIB strain of HIV-1 at various multiplicities of infection (MOI, calculated with perpheral mononuclear cells) in the presence of 2 μg of Polybrene per ml for 2 h at 37°C, using the procedure of Laurence et al. (9). The cells were then washed and resuspended in 1 ml of culture medium (RPMI 1640 containing 10% fetal bovine serum [Flow Laboratories] for U937 and the same components plus G418 for all transfected cells). The cells were plated in duplicate (0.5 ml per well). One half of the culture medium was removed and replaced with fresh medium every 3 or 4 days. At intervals postinfection, samples of these cells were tested for virus growth by assaying for p24 by enzyme-linked immunosorbent antigen capture as instructed by the manufacturer (DuPont). Cells were also tested for viability by using trypan blue dye exclusion. The levels of G418 used had no effect on susceptibility of cells to infection or level of p24 antigen produced.

RESULTS

Preparation of a multitargeting U1/HIV antisense construct. HIV-1 antisense sequences were inserted into the U1 transcript sequence as depicted in Fig. 1A. The 41 bp removed from the transcript region were replaced with the antisense oligonucleotides sequences A (68 bp), B (62 bp), C (61 bp), and D (63 bp). These insertions result in an increase of 27 bases in the length of the U1 transcript region of pDU1A, 21 bases in pDU1B, 20 bases in pDU1C, and 22 bases in pDU1D. The effect of these replacements on U1 RNA structure was assessed by the McDNASIS program (Hitachi, Inc.). The predicted secondary structures in all cases indicated that the insertions do not alter the secondary structure of the 3′ end; i.e., the U1/HIV antisense molecules are predicted to form normal stem-loops III and IV as well as a normal Sm region.

In situ assays for U1/HIV antisense RNA in 2.10.16 cells transfected with pNDU1A,B,C. To mimic more closely the expected therapeutic use of these constructs, a pool of transformed clones (designated 2.10.16) was obtained by transfection of U937 cells with plasmid pNDU1A,B,C and subsequent enrichment of the transfected population for G418 resistance (Materials and Methods). Figure 2 shows expression of the U1/HIV antisense sequences in 2.10.16 as analyzed by in situ amplification assay. These experiments used primers HU1-1 and HVA-2. Similar results were obtained when either HVB-2 or HVC-2 was used as the downstream primer. Note the nuclear localization of the dark blue precipitate indicative of the PCR-amplified cDNA (Fig. 2D). Nuclear staining (hematoxylin and eosin) indicates the large area of the cells occupied by the nucleus (Fig. 2A). It is evident that 10 to 20% of the 2.10.16 cells express transcripts corresponding to the U1/HIV inserts at a high enough level to be detected by this assay. 2.2.78 cells (containing the control plasmid) showed no signal (Fig. 2C), and omission of reverse transcriptase from the amplification procedure resulted in no signal in 2.10.16 cells (Fig. 2E).

HIV challenge of 2.10.16 cells. The 2.10.16 cells were then exposed to the BAL strain of HIV-1 at an MOI of 0.15. As can be seen in Table 1 (challenge 1a), this pooled clone 2.10.16 showed resistance to HIV-1, as indicated by a reduction in p24 production compared to the control clones (either U937 or

FIG. 1. Insertion of antisense (AS) sequences into U1 operons. (A) Hybridization of the deoxyoligonucleotide (Oligo) pairs resulted in double-stranded molecules with overhanging single-stranded termini compatible with the *BclI* and *Bsp*E1 ends produced from the restriction enzyme digestion of the U1 transcript region of the plasmid. The resulting plasmids containing the inserts were designated pDU1A, pDU1B, pDU1C, and pDU1D. (B) Assembly of a DNA construct (pNDU1A,B,C) containing three U1/HIV antisense cassettes.

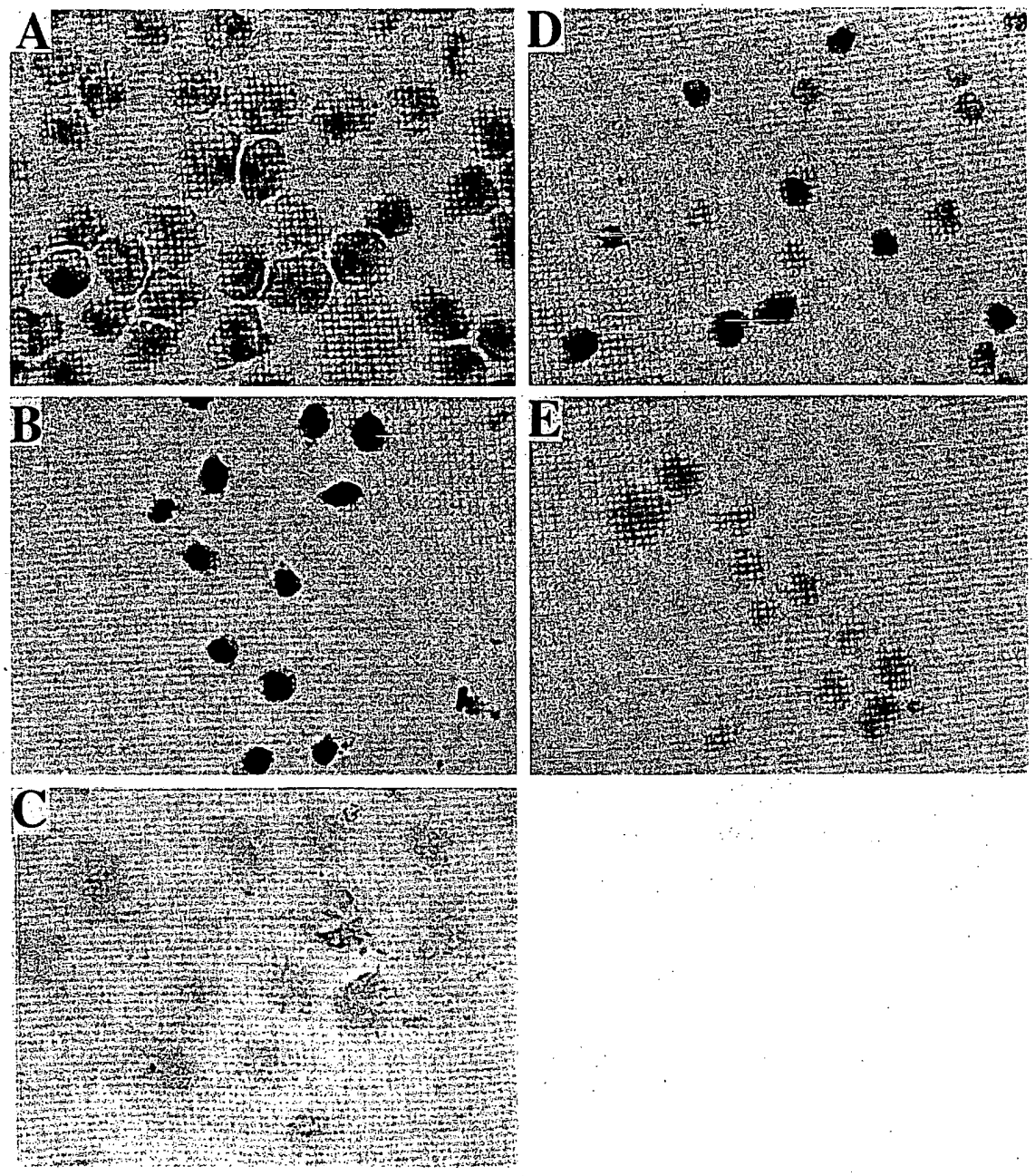


FIG. 2. In situ RT-PCR assay for U1/HIV antisense RNA in 2.10.16 (unchallenged) and 2.10.16R2 (challenged) cells. (A) Nuclear staining shows the cytologic features of the 2.10.16 cells after staining with hematoxylin and eosin. Note the large blue nucleus and the thin rim of pink cytoplasm. (B) In situ RT-PCR for U1/HIV antisense sequence A in 2.10.16R2 (postchallenge) cells revealing a strong nuclear signal in all cells. (C) In situ RT-PCR for U1/HIV antisense sequence A in 2.2.78 cells. (D) In situ RT-PCR for U1/HIV antisense sequence A in 2.10.16 (prechallenge) cells. Note that a minority of the cells have a detectable nuclear signal. (E) In situ RT-PCR for U1/HIV antisense sequence A in 2.10.16 cells when the reverse transcriptase treatment step is omitted.

2.2.78) at 6 days postinfection. In another challenge experiment (Table 1, challenge 1b), 2.10.16 cells showed greater than 90% inhibition of p24 production in the culture (compared with parent U937 cells and 2.2.78 cells) at 16 days postinfection. Cell viability assays performed on day 24 postinfection by using trypan blue exclusion showed that 17% of 2.2.78 pool controls from challenge 1b were viable. The pooled clone 2.10.16 were 40 to 60% viable and had no visible syncytia (data not shown).

At day 24, the 2.2.78 pool (which contains a DNA sequence unrelated to either HIV-1 or U1 inserted into the U1 transcript region [see Materials and Methods]) and the 2.10.16 cells were subjected to Ficoll gradient separation to separate live cells from dead cells, and the procedure was continued as a routine maintenance procedure as necessary until day 35. At day 35, there were no live cells remaining in the 2.2.78 culture.

Second challenge of 2.10.16 and U937 control cells. The pooled clone population 2.10.16 taken from day 31 postinfection (now termed clone 2.10.16R1) and U937 control cells (2.2.78) were subjected to a second challenge with the HIV-1 BAL strain at an MOI of 0.1. The cells were infected and maintained as before. Results of p24 assays at days 9 and 12

TABLE 1. HIV challenge of U937, 2.10.16, and 2.2.78 cells

Cells	p24 (pg/ml)		% Viable cells,[a] challenge 1b, day 24
	Challenge 1a, day 6	Challenge 1b, day 16	
U937	959	200	0
2.10.16 pooled clones	514 (46.6[b]) 554 (42.2)	12 (94.5)	40–60
2.2.78 pool control	780	220	17

[a] Assayed by trypan blue staining assay.
[b] Percent difference in amount of p24/ml when the 2.10.16R1 cell supernatant is compared with the supernatant from U937 cells.

postinfection are presented in Table 2. At day 12 approximately 66% inhibition of p24 antigen production was observed in 2.10.16R1 cells compared with U937 control cells. The cells were then maintained with 3- to 4-day Ficoll separation of the live from dead cells (as described above) until day 21. At this time, 2.10.16R1 showed no evidence of p24 antigen and the cells were 100% cell viable, as determined by trypan blue staining (data not shown). In contrast, 2.2.78 control cells (U937 control, Table 2) produced greater that 1,800 pg of p24 per ml, and parental U937 cells (not shown) weren't viable.

Third challenge of 2.10.16 cells. In this experiment, the pooled clone population identified as 2.10.16R1 from day 21 of the second challenge experiment (now referred to as clone 2.10.16R2) was infected with the IIIB strain of HIV-1 as described for the second challenge experiment. No p24 product was obtained following exposure of 2.10.16R2 cells to an MOI of 0.01, while high-level infection was seen in both parental U937 cells and control 2.2.78 cells.

In situ RT-PCR assay for U1/HIV antisense RNA in 2.10.16R2 (postchallenge) cells. In situ RT-PCR performed on the 2.10.16R2 cells showed that over 90% of these cells expressed U1/HIV antisense RNA at a detectable level (Fig. 2B), as determined by using HU1-1 (see Materials and Methods) as the upstream primer and either HVA-2 or HVC-2 as the downstream primer. Using HVB-2 or HVC-2 as the downstream primer gave similar results. The production of U1/HIV antisense in essentially all of the postchallenge cells is in contrast to a detection rate of 10 to 20% in prechallenge cells (Fig. 2D).

Tat-activated expression in cells transfected with U1/HIV antisense constructs. The effect of U1/HIV antisense constructs on Tat-activated expression was determined in transient expression assays measuring Tat-activated expression of CAT (see Materials and Methods). In unchallenged cells (Fig. 3A), whether clone 2.10.16, which carries the multitargeting construct pNDU1A,B,C), or any cells carrying the single targeting constructs (4.12, 5.34, or 8.54, carrying HIV antisense sequence A, B, or C, respectively), expression was inhibited. Postchallenge cells 2.10.16R2 (Fig. 3B) also demonstrated inhibition or Tat-activated CAT expression.

Assay for CD4$^+$ antigen of the surface of 2.10.16R2 cells. To determine whether the 2.10.16R2 cells had retained the CD4$^+$ antigen, these cells were examined in flow cytometry after treatment with mouse anti CD4$^+$ antibody (Leu-3a; Becton Dickinson) and fluoresceinated goat anti-mouse (Tago). The analysis showed that 45% of 2.10.16R2 cells contained CD4$^+$ antigen at a measurable level. This is equivalent to the 50 to 60% level normally observed in the parent U937 cells and indicates that the observed resistance to HIV-1 cannot be attributed to loss of the receptor protein CD4$^+$.

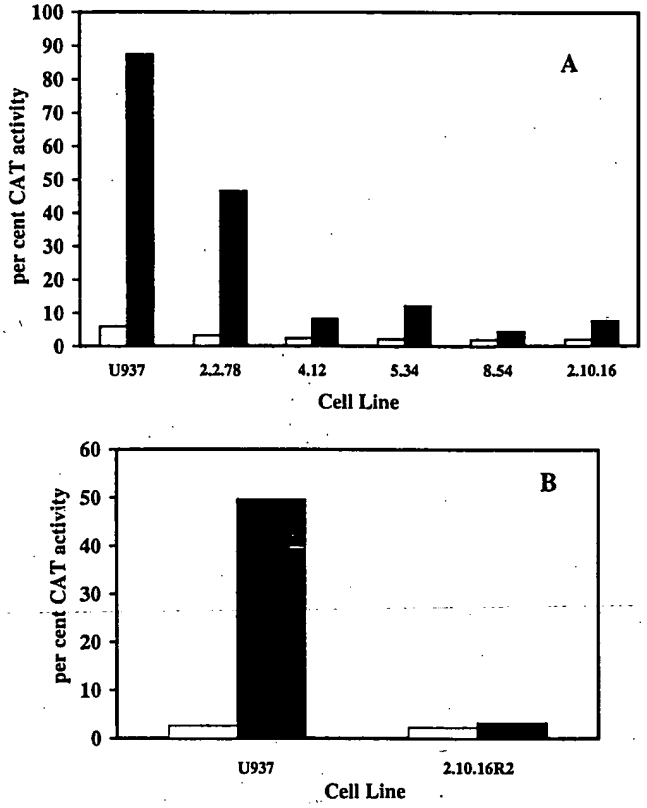


FIG. 3. Effect of U1/HIV antisense RNA on Tat-activated CAT expression. Transient expression assays for CAT were performed as described in Materials and Methods. Empty bars represent CAT expression in the absence of Tat; filled bars represent CAT expression in the presence of Tat. (A) CAT activity in prechallenge cells. The cell lines are described in the text. The 2.2.78 cells are transfected with the control plasmid pNDU1D. (B) CAT activity in postchallenge cells 2.10.16R2.

TABLE 2. Second HIV challenge of 2.10.16R1 and the parental U937 control cells

Cells	p24 (pg/ml)		
	Day 9	Day 12	Day 21
U937 control	3	5.1	>1,800
2.10.16R1	0	14.3	0

PCR assay for HIV-1 sequences in 2.10.16R2. Further evidence that the virus is not present in this cell population resulted from PCR assays for HIV nucleic acid. In assays using DNA PCR (Perkin-Elmer) and the manufacturer's primers which recognize the *gag* coding region of the BAL strain of HIV-1, no viral DNA was detected in 2.10.16R2 cells. As can be seen in Fig. 4A, by using various numbers of untreated U1.1A cells as a positive control, as few as 10^1 to 10^2 U1.1A cells can be detected. However, amplification products of DNA from 10^5 2.10.16R2 cells showed no HIV DNA. Similarly, PCR in situ hybridization using primers specific for the *gag* gene did not detect the provirus in any of the 2.10.16R2 cells. This is in contrast to a detection rate of over 90% in the HIV-infected pooled lymphocytes (data not shown).

PCR assay for presence of the U1/HIV antisense construct in 2.10.16R2 cells. 2.10.16R2 cells maintained for 60 days postchallenge were assayed for the presence of the U1/HIV antisense DNA by PCR assay. This assay used a primer pair representing U1 transcript region sequences which occur on either

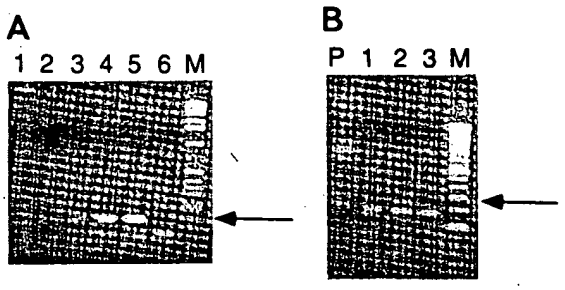


FIG. 4. (A) PCR assay for HIV-1 *gag* in HIV-infected control cells and in 2.10.16R1 cells and 2.10.16R2 cells. The arrow indicates the HIV-1 *gag* amplicon (147 bp). Lane 1, 10^2 U1.1A cells (promonocytic cells with two stably integrated copies of HIV); lane 2, 10^3 U1.1A cells; lane 3, 10^4 U1.1A cells; lane 4, 10^5 U1.1A cells; lane 5, 10^4 2.10.16R1 cells (from day 31 after initial HIV infection); lane 6, 10^5 2.10.16R2 cells (from day 60 after two challenges by HIV-1). (B) PCR assay for the pNDU1A,B,C sequences. The arrow indicates the amplicon for U1/HIV antisense sequences (199 to 206 bp). Lane P, amplification of a 1:1 mixture of DNA from pNDU1A,B,C and from pUC13 carrying the U1 operon; lane 1, amplification of DNA from 2.10.16R2 cells from 60 days (after two HIV-1 challenges); lane 2, amplification of DNA from 2, 2.10.16R1 cells from 31 days after the initial HIV-1 challenge; lane 3, amplification of DNA from U937 cells. Lanes M contain molecular weight markers.

side of the inserted antisense sequences. An amplicon derived from the U1 sequence (without an insert) would be 179 bp in length. An amplicon derived from U1 DNA with an inserted HIV antisense sequence would be 206 bp for sequence A, 200 bp for sequence B, and 199 bp for sequence C, or 20, 21, and 27 bp longer than the U1 amplicon, respectively. Figure 4B presents the results of amplification of 2.10.16R2 cells from day 60 postchallenge (lane 1) and 2.10.16R1 cells from day 31 postchallenge (lane 2). DNA derived from these cells yields two bands on electrophoresis (Fig. 4B). The upper band is approximately 200 bp and is absent in U937 cells (which contain no U1/HIV antisense sequences and only endogenous U1 gene sequences). The two bands are coincident with bands produced from an equal mixture of DNAs from pNDU1A,B,C and from pUC13 with the inserted U1 operon (lane P). These data are consistent with the presence of the U1/HIV antisense sequences in clones 2.10.16R1 and 2.10.16R2.

DISCUSSION

We report human HIV-1-resistant immune cells in culture that show a high level of resistance to multiple challenges by HIV for a prolonged period. During the challenge period, these cells maintained viability, showed no evidence of latent viral DNA, and retained their $CD4^+$ phenotype. These data demonstrate that HIV-1-resistant $CD4^+$ cells can be developed by using U1/HIV antisense constructs.

Attempts have been made by others to use genetic antisense constructs as a means of obtaining immune cells that are resistant to HIV-1 by utilizing antisense sequences against one specific HIV-1 target gene (2) or single RNA transcripts with antisense sequences against more than one HIV-1 target. In contrast to these studies, which showed a short period of HIV-1 resistance followed by a period of virus growth and ultimate cell destruction (6, 19), we have been successful in conferring a stable, HIV-1 resistance to immune cells in culture by combining independent multitargeting with the use of U1 snRNA as an antisense carrier.

The use of U1 snRNA provides a way to localize antisense transcripts in the cell nucleus as a means for delivery of anti-HIV antisense RNA to target sequences. While an active population of U1/antisense transcripts may be present in the cytoplasm at a concentration below the level of detection, the in situ analyses presented herein indicate that a vast majority of the U1/HIV antisense RNA is located in the nucleus. Nuclear location could be advantageous for effectiveness since higher concentrations of the antisense RNA can be maintained in the smaller volume of the nucleus, interactions with mRNA can occur prior to or during processing and translation, and there would be no possibility of competition with messenger-binding ribosomes. U1 snRNA also provides its inherent properties of stability and high level of transcription. Furthermore, several U1 antisense cassettes, each comprising approximately 750 bp, can be incorporated into a single vector.

To retain U1 snRNA's inherent properties for the delivery of antisense sequences, we attempted to maintain the original size as much as possible by using replacement of U1 sequences with antisense sequences. The resulting increases were only 20, 21, and 27 bases to the three U1/HIV antisense constructs. Although this substitution disrupts the formation of loops I and II, which are responsible for binding of U1-specific proteins, it allows exposure of the antisense sequences to potential target RNA molecules. Also, insertion near the 5′ end of the transcript reduces the likelihood of disrupting secondary structures that may be critical for binding proteins that are responsible for reimportation to the nucleus. In fact, computer predictions of secondary structures of the hybrid U1/HIV antisense RNA molecules show no perturbation of the Sm region or loops III and IV.

The U937 immune cells carrying U1/HIV antisense RNA (2.10.16) were obtained as a cell population enriched for resistance to G418. Since selection was made for G418, and not for U1/HIV antisense expression, this population of cells would be expected to contain cells that vary widely in antisense expression. This variation in expression among clones has been seen previously by Muller et al. (12) and by Szabo et al. (22), who introduced foreign genes into U937 cells as part of constructs expressing G418 resistance. Thus, it is not surprising that the first HIV-1 challenge resulted in virus growth in a portion of the heterogeneous 2.10.16 cell population. Evidence that this population is indeed heterogeneous is supported by in situ RT-PCR assays which show that approximately 10 to 20% of the cells produce detectable levels of each of the U1/HIV antisense RNAs. HIV-1 replication was significantly reduced in the second challenge, and during the third challenge no virus growth was detected as indicated by p24 assay.

The development of this resistance to HIV-1 correlated with the observation that essentially all 2.10.16R2 cells produced U1/HIV antisense RNA, as shown by in situ analysis. Further evidence that U1/HIV antisense RNA is responsible for this HIV-1 resistance was provided by transient expression assays for Tat-activated expression of CAT in 2.10.16R2 cells. Here, 2.10.16R2, compared with U937 cells and U937 cells carrying a control construct (2.2.78), markedly inhibited Tat activated expression.

Although the U1 antisense was designed to act in the nucleus, the inability to detect provirus sequences in postchallenge cells suggests the possibility that the U1/HIV antisense is capable of acting in the cytoplasm prior to HIV-1 integration. The presence of active antisense in the cytoplasm could result from the natural kinetics of U1 processing (23) wherein the U1/HIV antisense chimeric molecules are present in the cytoplasm prior to reimportation to the nucleus.

We have successfully achieved stable resistance to HIV in human immune cells in culture. Independent multitargeting was used to combat the variability and mutability of the virus. This approach also provided independent expression of each of the target sequences from an independent and specific pro-

moter, taking advantage of high rates of synthesis. The choice of U1 as an antisense carrier provided structural stability and nuclear localization. This successful approach in cell culture is being developed as means of achieving a high level of stable resistance in patient cells for the purpose of developing an ex vivo therapy for treating HIV infections.

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ENZO BIOCHEM EXPANDS HIV DEVELOPMENT PROGRAM

Initiates Collaboration with St. Luke's - Roosevelt Hospital Center

FARMINGDALE, NY, JULY 1, 1996 -- Enzo Biochem Inc. (ASE:ENZ) announced today that it has expanded its HIV therapeutic product development effort with the initiation of a collaborative program with Dr. David Volsky, Director of the Molecular Virology Laboratory at St. Luke's - Roosevelt Hospital Center, a teaching hospital of Columbia University's College of Physicians and Surgeons and one of the largest AIDS centers in the nation.

"The initiation of this collaborative effort is very exciting to us and represents the next phase in Enzo's program to develop specific DNA medicines based on our novel gene regulation and delivery technology," said Dr. Dean Engelhardt , Enzo's Senior Vice President of Research. "Because of the successes already achieved by Enzo scientists and collaborators in using this technology to inhibit viral replication in human cells, we are now moving towards the development of protocols to allow us to enter human clinical studies," continued Dr. Engelhardt .

The results of the first phase of Enzo's HIV preclinical therapeutic program that was conducted in collaboration with researchers at Cornell University Medical College will be presented at the XIth International Conference on AIDS in Vancouver, British Columbia next week.

Dr. Volsky and his laboratory will focus on the development of human clinical protocols aimed at a therapeutic treatment for HIV, in which cells are removed from the patient, altered ex vivo - outside the body - and reinfused into the patient. Dr. Volsky who is a Professor of Pathology at Columbia's College of Physicians and Surgeons is an expert on the biology of the AIDS virus, especially its replication. His laboratory has been a major contributor to research on HIV-1 growth, gene delivery systems and the identification of new virus targets for AIDS therapies. "We are extremely confident that this approach will result in the achievement of resistance to HIV that is clinically significant and are very pleased at the chance to participate in such a program," said Dr. Volsky.

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The approach for developing an AIDS therapeutic product combines Enzo's novel gene delivery system, **GenSert™**, with Enzo's gene regulation technology. Designed to prevent HIV from replicating inside cells, it is targeted to be the first commercial therapeutic product to emerge from Enzo's development efforts and to provide the medical community with a powerful new weapon in the fight against AIDS.

Enzo Biochem is engaged in the research, development and manufacture of innovative health care products based on molecular biology and genetic engineering techniques, and in providing diagnostic services to the medical community.

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